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RAPID CYCLE AMPLIFICATION FOR CONSTRUCTION OF COMPETITIVE TEMPLATES

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Abstract

The time required for DNA engineering techniques can be significantly reduced with rapid temperature cycling. By air heating and cooling of 10 nl samples in capillary tubes, 30 cycles of amplification can be completed in less than 15 min. Rapid cycle amplification can be used to construct internal competitive templates for quantitative PCR. In most cases, the natural amplicon and its primers are available. Insertions, deletions, or point mutations are introduced into the natural amplicon to allow differentiation of natural and control templates. Only 2 overlap primers are required to insert any sequence from an inner fragment into an outer fragment, with optional replacement of any sequence in the outer fragment. Competitive templates that differ in GC content can be used to distinguish PCR products based on melting curves monitored with fluorescence during PCR. Rapid cycle amplification was used to modify a natural template through insertion of another fragment having different GC content. Alternately, a portion of the natural template was replaced by another fragment. In addition to reducing time and reagent requirements, rapid cycling techniques increase amplification specificity. Rapid cycling is particularly advantageous when low volumes, small sample numbers, and multiple sequential amplifications are required.

Introduction

When multiple sequential PCR amplifications are required for DNA engineering, rapid amplifications can reduce a several-day process to less than a day. In particular, engineering an insertion requires multiple steps (1). A simple, robust insertion/replacement protocol that minimizes primer synthesis and the time required to generate a final product is demonstrated here with rapid temperature cycling.

Rapid Cycle DNA Amplification

Most instruments for temperature cycling are very slow compared to the times required for product denaturation, primer annealing, and polymerase action. Transition times between temperatures are long because of the thermal mass and sample geometry of most systems. With instrumentation limits removed, amplification yield and product specificity are optimal when denaturation and annealing times are less than 1 sec (2, 3). This finding may be surprising, but merely reflects the poor match between standard instruments for DNA amplification and the physical/enzymatic requirements of the process. That denaturation and annealing steps can be reduced to such short times is not widely recognized. Cycle times can be significantly shortened by turning the denaturation and annealing "plateaus" into temperature "spikes" (Figure 4.1). Whereas several seconds are required for the entire sample volume to reach equilibrium in conical plastic tubes, sample temperature vs. time plots in glass capillary tubes show sharp spikes at denaturation and annealing temperatures (3, 4). The physical (denaturation and annealing) and enzymatic (elongation) reactions of DNA amplification occur very quickly; with the proper instrumentation, amplification times can be reduced an order of magnitude from prevailing protocols.

Standard temperature cyclers for PCR complete 30 cycles in about 2-4 hours. Rapid cycle DNA amplification uses temperature cycles of 20-30 sec, allowing 30 cycles to be completed in 10-15 min (3, 4). These systems use capillary tubes as sample containers with hot air temperature control. Because of the low heat capacity of air and the thin walls and high surface area of capillary tubes, small volume samples can be cycled very rapidly. The use of capillaries with forced air heating allows precise control of sample temperature at a speed not possible with other designs. Commercial rapid temperature cyclers are available that minimize any perceived difficulty with working in capillary tubes (3).

Figure 4.1 compares 4 different sample temperature/time profiles and their resultant amplification products after 30 cycles. Profiles A and B were obtained on a standard heating block/microfuge tube system. The transitions between temperatures are slow and many nonspecific bands are produced. Some of the nonspecific bands can be eliminated by limiting the time at each temperature (A vs B) within the limits of the instrument. Profiles C and D were obtained with a rapid temperature cycler. Amplification is specific, and although yield is maximal in C (60 sec elongation time), the product band is easily seen in D (10 sec elongation time).

Competitive Templates for PCR Quantification

Competitive templates are commonly used as internal controls for PCR quantification (5). The control templates differ from the natural templates in some way that can be differentiated after amplification. If the amplification efficiency is the same for both templates, the relative concentration after amplification reflects the relative concentration before amplification. Since the original concentration of the control template is known, the concentration of the natural template before amplification can be determined.

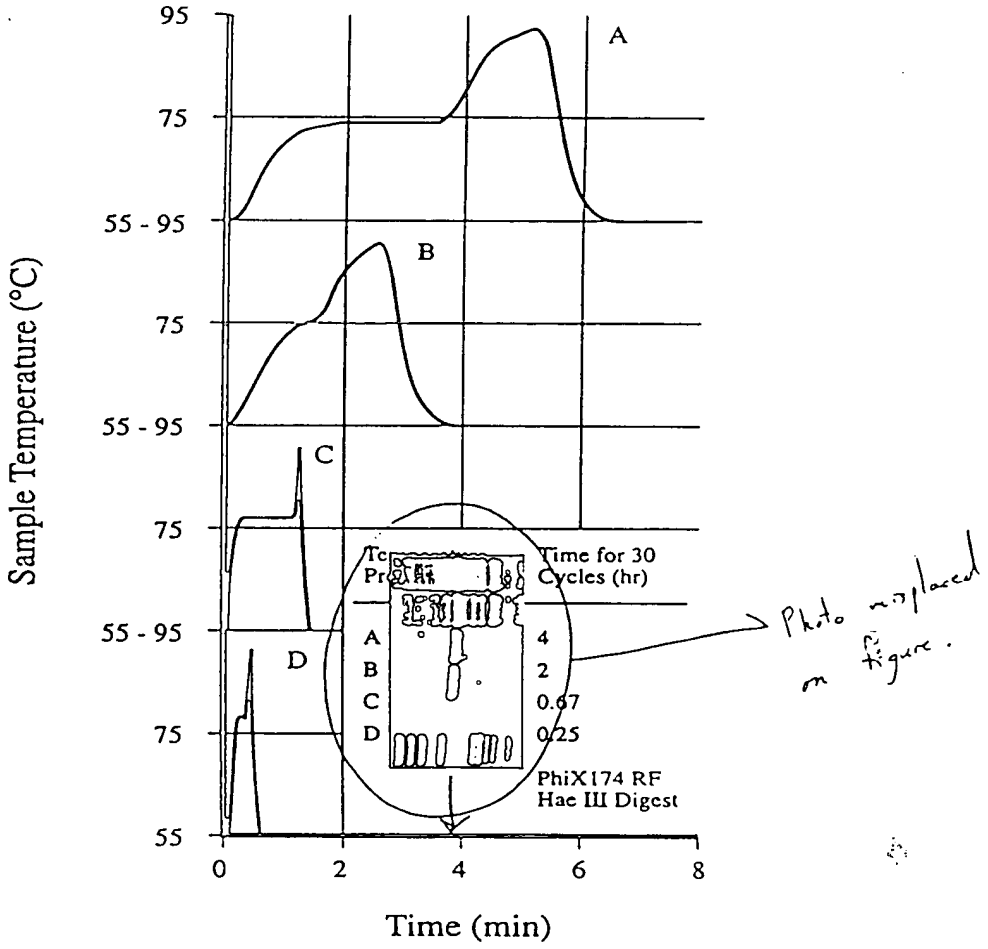

Figure 4.1. Comparison of temperature cycle profiles in a standard heat block instrument (A and B) and rapid cycling instrumentation (C and D). Temperature-time profiles and amplification products were obtained after 30 cycles of amplification of a 536 bp β-globin fragment from human genomic DNA. Ten µl samples were size-fractionated on a 1.5% agarose gel and stained with ethidium bromide. Sample temperature was monitored with a 0.2 mm diameter thermocouple probe (IT-23, Sensortek, Clifton, NJ). Profiles A and B were obtained with 100 µl samples in microfuge tubes overlaid with 60 µl of mineral oil as recommended by the manufacturer (Perkin-Elmer Cetus DNA Thermal Cycler, Norwalk, CT). In A, a typical protocol is shown (heating block at 93 °C for 1 min, 55 °C for 2 min, and 74 °C for 3 min). In B, the times were reduced so that the sample just momentarily reached denaturation and annealing temperatures (block at 92 °C for 35 sec, 55 °C for 35 sec, and 77 °C for 45 sec. Profiles C (1 min elongation) and D (10 sec elongation) were obtained with 10 µl samples in capillary tubes in a custom rapid air cycler. Reprinted with permission from BioTechniques (2)

Quantification by competitive amplification requires that the amplification efficiencies of the control and natural templates be known, and preferably equivalent. Usually, the control and natural templates are amplified with the same primers to eliminate differences in primer annealing and extension initiation. Similarly, the templates are usually kept around the same size to minimize differences in the completion of extension. When the amplified templates are separated on agarose gels, a minor difference in template size allows differentiation and seldom affects extension efficiency. Different templates of the same size can still be analyzed on gels if a sequence change incorporates a restriction enzyme site. After amplification, the products are digested with the restriction enzyme before electrophoresis.

When simple agarose gel electrophoresis is used to separate control and natural products, the templates are usually amplified well into the plateau phase. In this case, it is advantageous to keep the control and natural templates nearly identical (i.e. a single base change) so that efficient heteroduplex formation occurs. Double-stranded product formation during cooling largely determines the plateau effect of PCR (6). If the sequences of control and natural templates are almost identical, heteroduplex formation should occur at nearly the same rate as homoduplex formation and the amplification efficiency of both templates should decrease in parallel during the plateau phase. However, heteroduplexes may migrate differently than homoduplexes, complicating the gel analysis (5).

One way to assure accurate quantification with competitive templates is to determine the amplification efficiency of each template. This is now possible with continuous fluorescence monitoring using either double-strand-specific DNA dyes or sequence-specific resonance energy transfer probes (6-8). In addition, with the double-strand-specific DNA dye SYBR™ Green I, fluorescence melting curves can identify and quantify products differing in T_m during amplification (9). If a control template is engineered with a T_m different than the natural template, fluorescence analysis during temperature cycling could assess the relative amounts of both templates and determine the amplification efficiency of each. Control template T_m can be modified by changing the length or GC content of the natural template.

Insertion Method

The insertion design is diagrammed in Figure 4.2 and has been previously described (1). Given two DNA fragments, two overlap primers are required to define the ends of the inner amplicon and the position of insertion. Two additional primers specify the ends of the outer amplicon that harbors the insertion. The inner amplicon is first amplified with both overlap primers. Next, the inner amplicon is attached to each end of the outer amplicon in separate reactions by overlap extension and amplification with one outer primer and one overlap primer. The two intermediate products both contain the inner amplicon and are mixed, extended and amplified to yield the full length product.

Figure 4.2. Method for inserting an inner amplicon into an outer amplicon. The outer (1) and inner (2) amplicons are amplified from any source template (genomic DNA, plasmid, or longer amplicons). The outer amplicon (1) is obtained using primers O_1 and O_2, and contains contiguous attachment sequences A_1 and A_2. The inner amplicon (2) is obtained using primers I_1 and I_2. However, primers I_1 and I_2 are 5' extended by attachment sequences A_1 and A_2 so that the inner amplicon includes the sequences A_1I_1 and A_2I_2 at its termini. The inner and outer amplicons are gel purified (1.5% agarose), the agarose dissolved, and the DNA absorbed and eluted from a silica matrix (QIAquick gel extraction kit, Qiagen, Chatsworth, CA). In 3a, the A_1 sequence of the outer amplicon anneals to the complementary A_1' sequence of the inner amplicon. The A_1' sequence terminates at a free 3' end that serves as a primer for overlap extension. Primers O_1 and A_2I_2 exponentially amplify the product $O_1...A_1I_1...I_2'A_2'$. In 3b, the A_2 sequence of the outer amplicon anneals to the complementary A_2' sequence of the inner amplicon. The A_2' sequence terminates at a free 3' end that serves as a primer for overlap extension. Primers O_2 and A_1I_1 exponentially amplify the product $O_2...A_2I_2...I_1'A_1'$. The overlap-extension products are gel purified as above. In 4, combination of the two products from steps 3a and 3b provides free 3' ends at the termini of A_1' and A_2' that serve as primers for overlap extension. Primers O_1 and O_2 exponentially amplify the final product in which the inner amplicon from step 2 has been inserted into the outer amplicon from step 1.

should be ① , not ②

Insertion Protocol

A 193 bp, low GC fragment from exon 10 of the human cystic fibrosis gene was inserted into a 239 bp, high GC fragment of the 5' untranslated region of the hepatitis C genome. Five amplifications were required (Figure 4.2). Rapid cycle DNA amplification was performed as previously described (2, 3) in 10 µl capillary tubes with 50 mM Tris, pH 8.3, 3 mM $MgCl_2$, 0.2 mM each dNTP, 0.5 µM each primer, sucrose (2% w/v), cresol red (0.1 mM), bovine serum albumin (500 µg/ml), 0.4 U of native *Taq* polymerase/10 µl and variable amounts of template as described below. Half of the bovine serum albumin (BSA) was included in a dilution buffer for the polymerase (250 µg/ml BSA, 10 mM Tris, pH 8.3) so that a 10X polymerase solution (0.4 U polymerase/µl) could be made from 5U/µl stocks. The remaining BSA and the Tris, $MgCl_2$, sucrose, and cresol red were included in a 10X buffer. The dNTP mixture, primers, and template DNA were also used as 10X solutions so all reagents were added as 1 µl additions for each 10 µl reaction. Reagent solutions (except template) are available commercially (Idaho Technology, Idaho Falls, ID).

A rapid air thermocycler was used for all amplifications (RapidCycler™, Idaho Technology). The annealing to extension temperature slope was always 3 °C/sec. The denaturation and annealing times were for "0" sec., meaning the temperature extremes were attained but only momentarily held.

Formation of the Outer Amplicon

A 239 bp amplicon of the hepatitis C genome was amplified from a larger amplicon that was previously obtained by PCR amplification of the cDNA from a clinical isolate. The primers were O_1 (CGTCTAGCCATGGCGTTAGT) and O_2 (GCACTCGCAAGCACCCTATC) and 1 µl of a 10^{-6} dilution of the larger amplicon was used without purification. Thirty cycles of denaturation at 94 °C for 0 sec, annealing at 55 °C for 0 sec, and extension at 74 °C for 5 sec required 13 min. The 10 µl product was sized fractionated on a 1.5% agarose gel, purified with a commercial kit (QIAquick gel extraction kit, Qiagen, Chatsworth, CA) and eluted in 50 µl of 10 mM Tris, pH 8.0, 0.1 mM EDTA.

Formation of the Inner Amplicon with 5' Overlaps to the Outer Amplicon

A 233 bp amplicon including 193 bps of the cystic fibrosis gene was amplified from human genomic DNA. The overlap primers were A_1I_1 (ccgggagagccatagtggtcGACTTCACTTCTAATGATGA) and A_2I_2 (gtgtactcaccggttccgcaCTCTTCTAGTTGGCATGCTT), where the overlaps for attachment to the outer fragment are indicated in small letters, and correspond to adjacent regions on complementary strands. Fifty ng of template DNA (1 µl of DNA with an A_{260} of 1.0) were used. Ten cycles of denaturation at 94 °C for 0 sec, annealing at 45 °C for 0 sec, and extension at 74 °C for 5 sec were followed by 25 cycles of denaturation at 94 °C for 0 sec, annealing at 55 °C for 0 sec, and extension

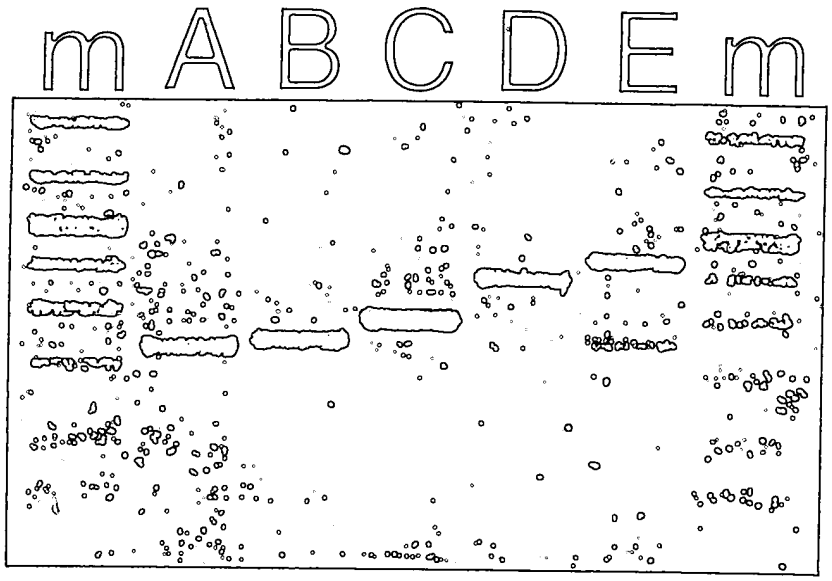

Figure 4.3. Insertion. Gel electrophoresis of original, intermediate and final products before purification. After amplification, 5 µl of each product was size-fractionated on 1.5% agarose gels in the presence of 0.5 µg/ml ethidium bromide. The products displayed are: A), the original inner amplicon comprising 233 bps from the human cystic fibrosis gene; B), the original outer amplicon comprising 239 bps of the hepatitis C genome; C), a 286 base-pair intermediate overlap-extension product amplified with primers O_1 and A_2I_2; D), a 379 base-pair intermediate overlap-extension product amplified with primers O_2 and A_1I_1, and E) the 432 base-pair final product. Some 239 base-pair outer amplicon can be detected along with the final 432 base-pair product in lane E. The outer two lanes contain DNA marker bands of 1000, 700, 500, 400, 300, 200, 100 and 50 bps (BioMarker™ Low, BioVentures, Murfreesboro, TN).

at 74 °C for 5 sec. The amplification required 18 min. Ten µl of product was size fractionated, purified, and eluted as above.

Formation of the Intermediate Overlap Products

The inner and outer amplicons were combined and 1 µl of the mixture used as template in 2 different reactions to form the intermediate overlap products. Primers O_1 and A_2I_2 were used to attach the inner amplicon to one strand of the outer amplicon, while primers O_2 and A_1I_1 were used to attach the inner amplicon to the complementary strand. For both reactions, 2-temperature cycling was used with 30 cycles of denaturation at 94 °C for 0 sec and a combined annealing/extension phase at 68 °C for 20 sec for a total cycling time of 18 min. Ten µl of each product was size fractionated, purified, and eluted as above.

Formation of the Final Product

The 2 intermediate overlap products were combined and 1 µl of the mixture used as template in the final reaction. A single initial overlap extension step was performed without primers by denaturation at 94 °C for 0 sec and extension at 70 °C for 120 sec. The capillary was opened and the 9 µl solution mixed with 1 µl of a 10X solution of primers 0_1 and 0_2. The sample was aspirated into the original capillary, resealed and temperature cycled 20 times at 94 °C for 0 sec, 55 °C for 0 sec, and 74 °C for 10 sec. Overlap extension and amplification required 13 min.

Agarose gel electrophoresis of all products is shown in Figure 4.3. The specificity of each amplification is good, although some original outer product can be detected in the final amplification.

Method for Replacements, Small Insertions, and Deletions

The general insertion method is very flexible and powerful. The inner and outer amplicons can be selected from any region of existing DNA and any attachment point can be chosen. If a replacement is desired instead of an insertion, the attachment sequences in the outer amplicon are separated (Figure 4.4A) so that the intervening sequence is deleted when the insertion method is followed (Figure 4.2). In the final product, the inner amplicon replaces the region of the outer amplicon that lies between the attachment sequences.

When the sequence being inserted is greater than 30-40 bases, 2 overlap primers and an external template are needed (Figure 4.2). When less than 35 bases are being inserted, no external template for formation of the inner amplicon is needed (Figure 4.2, step 2). Instead, the inner amplicon can be formed directly as a primer dimer with the insertion bases sandwiched between attachment sequences (Figure 4.4B). The intermediate overlap and final products are then formed as in Figure 4.2 (3a, 3b, and 4).

When the sequence being inserted is less than 10 bases, only a single overlap primer of less than 50 bases is needed (Figure 4.4C). The single primer includes the insertion bases between both attachment sequences. When the overlap product (Figure 4.4C) is purified and combined with the outer amplicon and primer O_1, the final insertion or replacement product is formed. If the single overlap primer does not include insertion bases (Figure 4.4D) and the attachment sequences are separated (Figure 4.4A), a deletion occurs. In summary, replacements, insertions or deletions of any size can be obtained.

Replacement

The 193 bp fragment from the human cystic fibrosis gene was used to replace a 76 bp fragment lying between separated attachment sequences in the 239 bp fragment of the hepatitis C genome. The insertion protocol was used but primer A_2I_2 was

Figure 4.4. Method for replacements, small insertions, and deletions. For replacements, the attachment sequences A_1 and A_2 are separated in the outer amplicon (Diagram A, compare to Diagram I in Figure 4.2). When the insertion protocol diagrammed in Figure 4.2 is followed, the inner amplicon will replace the fragment of the outer amplicon that lies between A_1 and A_2. Insertions of less than 35 bases can be constructed without exogenous template via a primer dimer (Diagram B). The primers include the insertion sequence (I) and the primer dimer formed serves as the inner amplicon of Figure 4.2. Insertions of less than 10 bases require only a single primer that contains the insertion bases (I) flanked by the attachment sequences, A_1 and A_2 (Diagram C). The intermediate overlap product formed from Diagram C is purified and overlap extended in the presence of the outer amplicon and primer O_1 to form the final desired product. If the initial overlap primer contains both attachment sequences but no insertion bases (Diagram D), a deletion will result if the attachment sequences are separated in the outer amplicon (Diagram A).

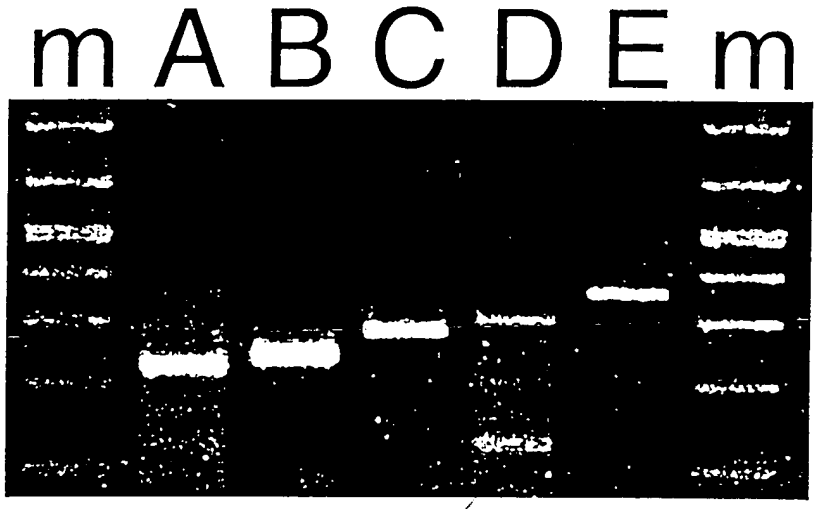

Figure 4.5. Replacement. Gel electrophoresis of original, intermediate and final products before purification. After amplification, 10 µl of each product was size-fractionated on 1.5% agarose gels in the presence of 0.5 µg/ml ethidium bromide. The products displayed are: A), the original inner amplicon comprising 233 bps of the human cystic fibrosis gene; B), the original outer amplicon comprising 239 bps of the hepatitis C genome; C), a 286 base-pair intermediate overlap-extension product amplified with primers O_1 and A_2I_2; D), a 303 base-pair intermediate overlap-extension product amplified with primers O_2 and A_1I_1; and E), the 356 base-pair final product. The outer two lanes contain DNA marker bands of 1000, 700, 500, 400, 300, 200, 100 and 50 bps.

modified (gcgggggcacgcccaaatCTCTTCTAGTTGGCATGCTT) so that attachment sequences A_1 and A_2 were separated by 76 bps in the hepatitis C genome (Figure 4.4, A). Agarose gel electrophoresis of all products is shown in Figure 4.5.

Guidelines for Optimization of Rapid Cycle PCR

Using minimal denaturation and annealing times in rapid cycling helps simplify optimization. The denaturation and annealing times are always kept at "0" sec, so there are only 5 parameters to vary (denaturation temperature, annealing temperature, extension temperature, extension time, and number of cycles) instead of 7. The choice of the remaining 5 parameters depends on 1) the product T_d and T_m, 2) the primer T_m's, 3) the product length, and 4) the starting template concentration.

Product melting temperatures can vary over a 50 °C range depending on GC content and length (9). Product melting affects both the required denaturation temperature and acceptable extension temperatures. The denaturation temperature in PCR must be greater than the strand dissociation temperature of the product, T_d. Usually, a temperature between 90 °C and 94 °C is chosen as a convenient compromise between increased enzyme denaturation at higher temperatures and concern over not melting the product at lower temperatures. In practice, products <75% GC usually melt below 94 °C and 94 °C is commonly used as a default denaturation

Table 4.1. Suggested temperature and time parameters for rapid cycle DNA amplification

	Temperature (°C)	Time (sec)
Denaturation	94[a]	0 sec
Annealing	30 + 0.5(primer GC%)[b]	0 sec
Extension	74[c]	0.03 (product length)[d]

[a]for products with a high GC domain, consider adding DMSO, formamide, and/or increasing the temperature.
[b]data extracted from (12).
[c]may need to be lower for products with a high AT domain.
[d]for products < 100 base pairs, use a 0 sec extension time.

temperature. For products with a higher GC content, such as the fragile X repeat (10), DMSO or formamide can be added, the salt concentration can be lowered, and/or the temperature can be increased. In any case, extended denaturation times (denaturation times greater than 0 sec) are not helpful. Product melting may also limit acceptable extension temperatures. Polymerization rates of *Taq* increase with temperature up to about 80°C, allowing faster cycling with higher extension temperatures. However, the extension temperature must be less than the product T_m, or the polymerase may dissociate from the DNA instead of continuing to extend. This is a concern with low GC products, such as the variable repeat linked to the apolipoprotein B gene (11), where extension temperatures cannot be increased much above 65°C.

The T_m's of the primers determine acceptable annealing temperatures. Although many commercial programs can estimate primer T_m's and suggest appropriate annealing temperatures, some empirical tuning is usually necessary because these programs seldom adequately account for: 1) salt concentrations (KCl, Mg^{++}, dNTPs), 2) additives such as formamide and DMSO, and 3) variations in temperature cycling. With conventional slow cycling and long annealing times, an annealing temperature several degrees above the primer T_m may be appropriate, while with rapid cycling, a temperature nearer the T_m may be required. The following "rule of thumb" between rapid cycling annealing temperature and GC content of the primer may be useful: 45 °C for 30% GC, 50 °C for 40% GC, 55 °C for 50% GC, 60 °C for 60% GC, and 65 °C for 70% GC. Under these conditions, amplification is (almost) always successful somewhere between 1 and 4 mM Mg^{++}. With gel purification of intermediate engineered products, no time is lost if a range of Mg^{++} concentrations is tried and an acceptable product band is obtained.

The product length determines the required extension time. At an extension temperature of 70-74°C, the following guidelines can be used to set extension times according to product length: "0" sec for <100 bps, 5 sec for 100-200 bps, 10 sec for 200-400 bps, 15 sec for 400-600 bps, 20 sec for 600-800 bps, 30 sec for 800-1200 bps, and an additional 30 sec for each kilobase thereafter. The starting copy number and efficiency of amplification determine the number of cycles required. Table 4-1 summarizes the above optimization guidelines for 3-step cycling.

With rapid cycle amplification, 3-step temperature cycling is usually faster than 2-step cycling. This is because the annealing and extension conditions can be individually optimized in 3-step cycling and the extension temperature chosen for maximal extension rates. Two-step rapid cycle amplification is also effective (e.g., the intermediate overlap products shown in Figure 4.3, lanes C and D). However, optimization guidelines for the combined annealing/extension step of 2-step rapid cycling have not been derived.

Discussion

Rapid cycle DNA amplification can decrease the time required for DNA engineering techniques such as insertion protocols that require multiple sequential amplifications. The total amplification time for the 4 sequential amplifications for insertion or replacement was about 1 hour. Gel electrophoresis (3 x 30 min) and purification from the gels (2 x 30 min) required another 2.5 hours, allowing the entire procedure to be completed in half of a working day. Gel electrophoresis allows sequential monitoring of the construct, and gel purification increases the product purity at each stage.

One disadvantage of this method is that gel purification requires a difference in size to be effective. If the outer amplicon is the same size as one of the intermediate overlap products, the outer amplicon will be present in the final amplification and will compete with the desired final product. In this case, reamplification of a 10^{-6} dilution of the intermediate product will be more effective than gel purification in reducing the amount of outer amplicon. If the outer amplicon is near the same size as the final product, gel electrophoresis cannot be used to confirm successful modification. In practice, it may be difficult to completely eliminate the outer amplicon (Figure 4.3, lane E), and reamplification after gel purification of the final product may be required. Despite these limitations, the procedure is simple and rapid, and should work well in nearly all cases.

Part of the robustness of the method is attributable to rapid cycle amplification. Minimizing annealing times increases specificity (2, 3), and limiting denaturation times minimizes degradation (13). In addition to specificity advantages, it is easier to optimize rapid cycle amplifications than conventional slower procedures. When rapid cycling was used for allele-specific amplification, the annealing temperature could be varied over a 10-15 °C range, while only a 1-2 °C range was acceptable with slower, conventional cycling (4).

An interesting possibility for automatic optimization uses fluorescence feedback to control amplification parameters. For example, temperature cycling can be terminated when the amplification plateau is detected with fluorescent dyes (6-9). In addition, with a double-strand-specific DNA dye, the denaturation temperature can be dynamically set by increasing the temperature each cycle only until denaturation occurs. Similarly, the extension step of each cycle could be terminated when double-strand fluorescence plateaus or when a certain amplification efficiency is attained. Rather than empirical algorithms, the best solution to determining primer and product T_m's is to measure them, now routinely possible with these fluorescence techniques (6-9).

Reagent requirements for rapid cycling in capillary tubes are somewhat different from amplifications in conventional microfuge tubes. Bovine serum albumin needs to be included in the buffer to prevent surface inactivation of the polymerase on the glass capillary surface. Magnesium concentrations are usually higher than with conventional cycling to increase primer annealing rates. A "Rapid Cyclist User's Group" maintains a web site with helpful hints, protocols, and applications (http://128.110.64.121).

Rapid cycle DNA amplification is rapid, inexpensive and convenient. The perception that capillary tubes are difficult to work with quickly disappears with experience. High-density additives and electrophoresis indicator dyes are often included in the reaction buffer to simplify gel loading directly out of capillary tubes (2, 14). Reagent costs are minimized with small volumes. In general, rapid cycle DNA amplification can be used in any construction technique, but is particularly advantageous when multiple sequential amplifications are required.

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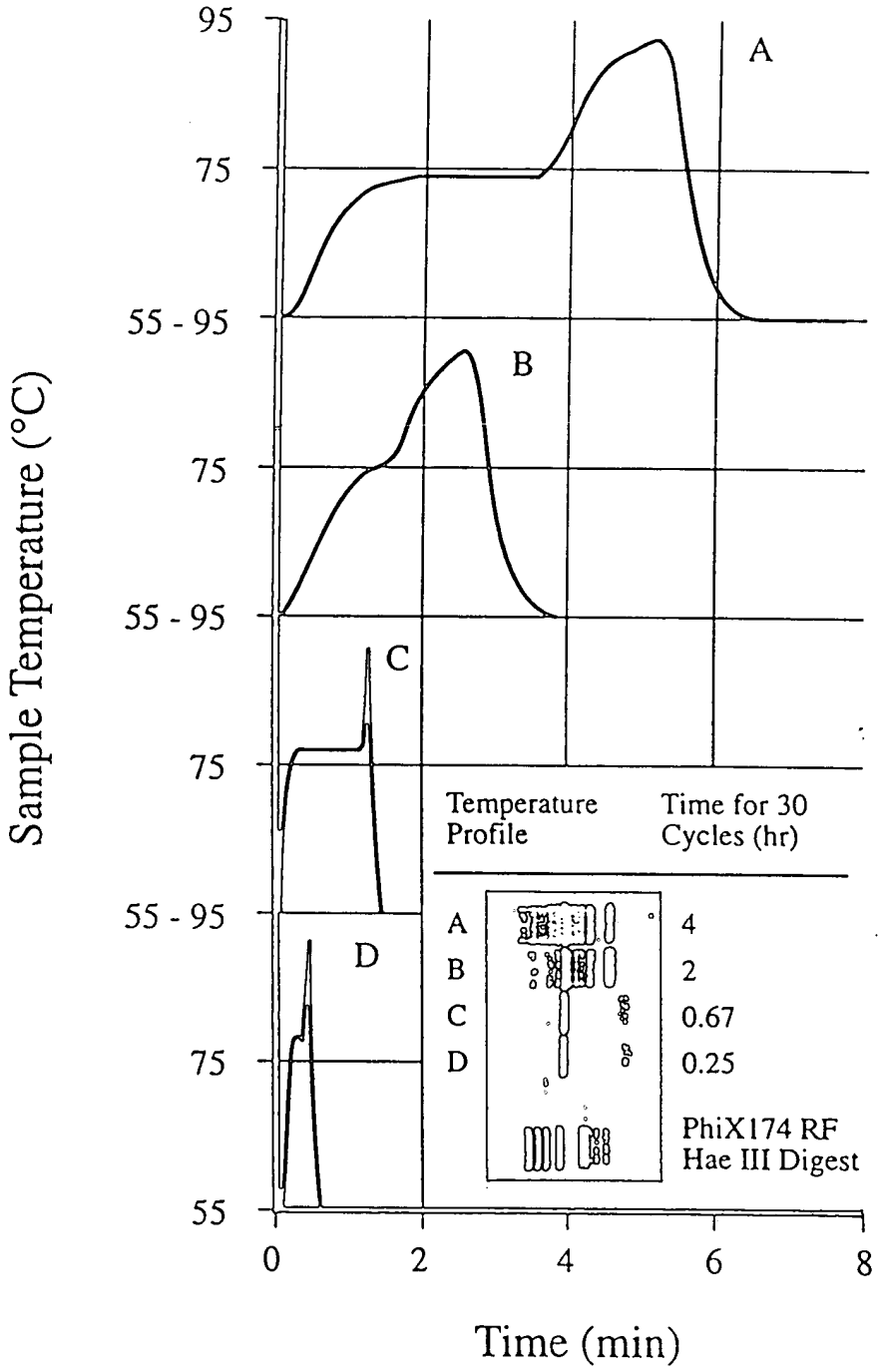
Sample Temperature (°C)
95
75
55 - 95
75
55 - 95
75
55 - 95
75
55
A
B
C
D
0
2
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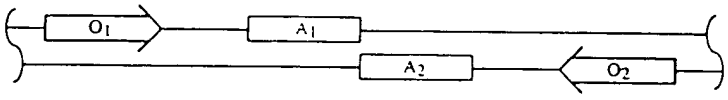
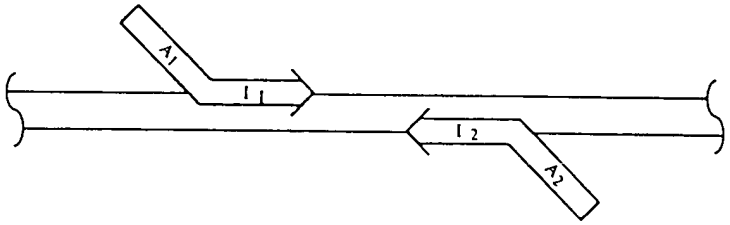
6
8
Time (min)
Temperature Profile
Time for 30 Cycles (hr)
A 4
B 2
C 0.67
D 0.25
PhiX174 RF Hae III Digest

1. Outer Amplicon



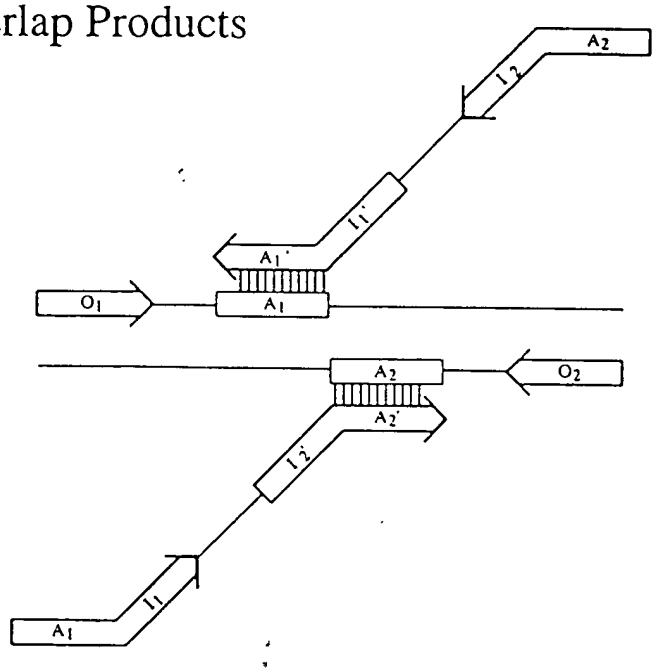
2. Inner Amplicon



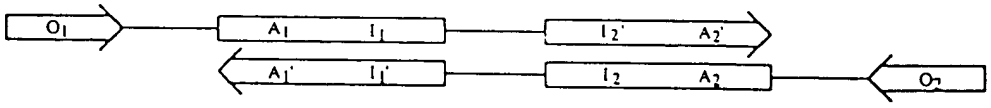
3. Intermediate Overlap Products

a.

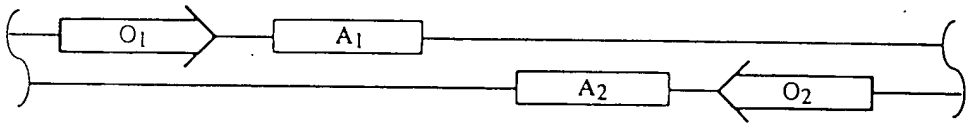
b.



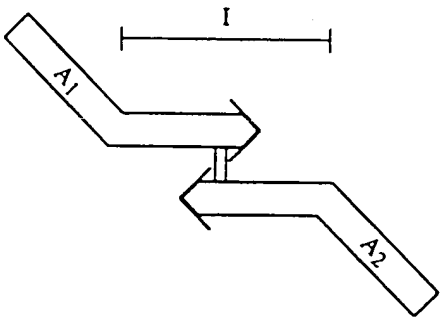
4. Final Insertion Product



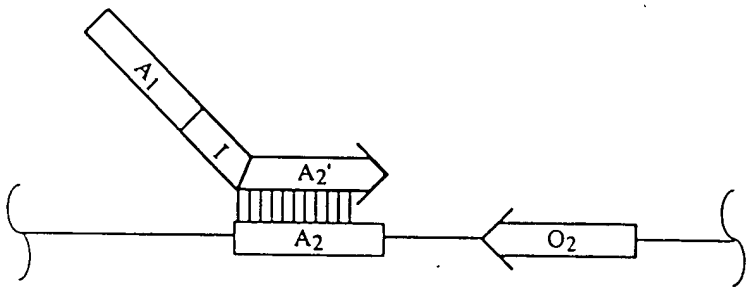
A. Replacements



B. Insertions <35 bases



C. Insertions <10 bases



D. Deletions